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EFFECT OF MELATONIN ON MOTOR PERFORMANCE AND BRAIN CORTEX MITOCHONDRIAL FUNCTION DURING ETHANOL HANGOVER

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Abstract—Increased reactive oxygen species generation and mitochondrial dysfunction occur during ethanol hangover. The aim of this work was to study the effect of melatonin pretreatment on motor performance and mitochondrial function during ethanol hangover. Male mice received melatonin solution or its vehicle in drinking water during 7 days and i.p. injection with EtOH (3.8 g/kg BW) or saline at the eighth day. Motor performance and mitochondrial function were evaluated at the onset of hangover (6 h after injection). Melatonin improved motor coordination in ethanol hangover mice. Malate–glutamate-dependent oxygen uptake was decreased by ethanol hangover treatment and partially prevented by melatonin pretreatment. Melatonin alone induced a decrease of 30% in state 4 succinate-dependent respiratory rate. Also, the activity of the respiratory complexes was decreased in melatonin-pretreated ethanol hangover group. Melatonin pretreatment before the hangover prevented mitochondrial membrane potential collapse and induced a 79% decrement of hydrogen peroxide production as compared with ethanol hangover group. Ethanol hangover induced a 25% decrease in NO production. Melatonin alone and as a pretreatment before ethanol hangover significantly increased NO production by nNOS and iNOS as compared with control groups. No differences were observed in nNOS protein expression, while iNOS expression was increased in the melatonin group. Increased NO production by melatonin could be involved in the decrease of succinate-dependent oxygen consumption and the inhibition of complex IV observed in our study. Melatonin seems to act as an antioxidant agent in the ethanol hangover condition but also exhibited some dual effects related to NO metabolism.

Key words: melatonin, ethanol hangover, motor coordination, mitochondrial function, mitochondrial membrane potential, oxidative stress.

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) is a highly conserved hormone mostly secreted by the pineal gland during the night in all known mammals including humans (Cardinali and Pevet, 1998).

Melatonin and its metabolites have been proven to be endogenous free-radical scavengers and broad-spectrum antioxidants (Tan et al., 2000; Manda et al., 2007; Reiter et al., 2008). Moreover, melatonin prevents neuronal cell death in a large number of models of brain damage where oxidative stress is involved, protecting for instance against seizures induced by kainate, glutamate, and *N*-methyl-D-aspartate (NMDA) (Lapin et al., 1998) and acting as a neuroprotector and an antioxidant agent both *in vivo* and *in vitro* (Cagnoli et al., 1995; Giusti et al., 1996). Moreover, effects of melatonin on rodent behavior have also been demonstrated; it induces sedation, elevates the threshold of pain perception, exhibits anxiolytic effects and directly resets circadian rhythms (Golombek et al., 1996).

Because of its small size and amphiphilic nature, melatonin easily reaches all cellular and subcellular compartments including mitochondria (Menendez-Pelaez and Reiter, 1993). Most of the beneficial consequences resulting from melatonin administration may depend on its effect on mitochondrial physiology (Jou et al., 2005, 2007; Peng et al., 2006). Melatonin's functions as an antioxidant include direct free radical scavenging, stimulation of antioxidant enzymes, increment of mitochondrial efficiency in relation to oxidative phosphorylation, enhancement of the electron transport chain activity and exacerbation of the effect of other antioxidants (Carpentieri et al., 2012).

Reactive oxygen species are generated during ethanol metabolism, causing oxidative stress and lipid peroxidation in brain (Calabrese et al., 1998; Comporti et al., 2010).

Ethanol hangover begins when alcohol disappears from blood and is characterized by unpleasant physical and psychological symptoms in humans (Kim et al.,

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Abbreviations: BAC, blood alcohol concentration; $DiOC_6$, 3,3″-dihexyloxacarbocyanine iodide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; H_2O_2, Hydrogen peroxide; HEPES, hydroxyethyl piperazineethanesulfonic acid; HRP, horseradish peroxidase; iNOS, Inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; L-NNA, Nω-nitro-L-arginine; NO, nitric oxide; O_2^-, superoxide anion; RCR, respiratory control ratio; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

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2003). Impairment in body temperature, wheel-running activity and pain perception has been described in experimental animals associated with this physiopathological state (Brasser and Spear, 2002; Varlinskaya and Spear, 2004). Results from our laboratory demonstrated that 6 h after an acute ethanol exposure, mice exhibited a reduction in motor performance associated with brain cortex mitochondrial dysfunction at the onset of ethanol hangover (Bustamante et al., 2012).

Other researchers reported that melatonin could prevent oxidative damage and mitochondrial DNA (mtDNA) depletion induced by acute ethanol administration (Mansouri et al., 2001). The aim of this work was to study the melatonin effect on motor performance and mitochondrial function during the ethanol hangover in male mice.

EXPERIMENTAL PROCEDURES

Materials

BSA, catalase, cytochrome c, dithiothreitol, EGTA, Folin reagent, glutamate, HEPES, L-arginine, malate, mannitol, Nicotinamide adenine dinucleotide (NADH), Nicotinamide adenine dinucleotide phosphate (NADPH), Nω-nitro-L-arginine (L-NNA), oxyhemoglobin, succinate, superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The potentiometric probe, 3,3″-dihexyloxacarbocyanine iodide ($DiOC_6$), was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals

Swiss mice (*Mus musculus*) weighing 30–40 g housed in a soundproof room, with humidity and-controlled temperature (22 ± 2 °C) with a 12:12-hour light:dark cycle photoperiod (lights on 0700 h), fed standard rat chow and tap water ''*ad libitum*'' were used. Animal handling and treatment, as well as all experimental procedures were reviewed in accordance with the guidelines of the National Institute of Health (USA), and with the 6344/96 regulation of the Argentinean National Drug Food and Medical Technology Administration (ANMAT). All efforts were made to minimize suffering and reduce the number of animals used.

Methods

Solutions preparation, melatonin exposure and ethanol injections. Ethanol (EtOH), 15% w/v, was prepared by diluting a 95% stock solution of EtOH with 0.9% saline solution (SS). Melatonin (Mel) (Sigma–Aldrich, St. Louis, USA) (purity: 982 mg/g) solution was solubilized in 10% ethanol and mixed in tap water (25 μg melatonin/ml), according to previous research (Kim et al., 2000a,b; Bruno et al., 2005). The final EtOH concentration in melatonin solution was 0.1%. Mice received melatonin solution or its vehicle in drinking water during 7 days during 24 h. At the eighth day, animals received an injection (i.p.) of EtOH (3.8 g/kg BW) or saline (9:00 am). The ethanol dose applied in this work was previously used in other studies (Gilliam et al., 1990; Mollenauer et al., 1992; Brasser and Spear, 2002; Fee et al., 2004). In order to ensure the stability of the melatonin solution and properly control its administration to mice, melatonin solution was prepared every day and the bottles containing drinking water were covered to prevent drug degradation by light. Motor performance was evaluated in the early afternoon (3:00 pm) at the onset of ethanol hangover. According to previous researches, ethanol hangover onset was considered 6 h after ethanol injection when blood alcohol concentration (BAC) was close to zero (Bustamante et al., 2012). Melatonin treatment during 7 days before ethanol exposure did not modify BAC levels (data not shown).

Evaluation of melatonin effect on motor performance during ethanol hangover. Motor coordination was evaluated with a modified tightrope test (Boveris and Navarro, 2008). Briefly, the procedure consisted of placing the animal on the middle of a 60-cm long horizontal rope suspended 30 cm above the floor and time was recorded until the animal either reached the end of the rope or fell down during a period of 60 s. A score was assigned accordingly: animals reaching the end of the rope in ⩽6 s were given 1 point and an additional point was given for every additional 6 s needed to complete the test. Animals that stayed on the rope for 60 s without reaching the end were given 11 points. When mice fell down, while the test was running, 11 points were assigned and 1 extra point was added for every 6 s before the test ending time (60 s). The test evaluates the motor performance of the animal as a mean of its intrinsic neuromuscular coordination. For this work, results were shown as a percentage of the motor performance which was calculated considering the maximum score for the test and the score reached for each animal.

Evaluation of melatonin effect on brain cortex mitochondrial function during ethanol hangover

Isolation of mice brain cortex mitochondria. As for the first experiment, animals received melatonin or vehicle during 7 days and, at the eighth day received an i.p. injection of ethanol or saline. Six hours after injection, animals were killed by cervical dislocation in accordance with the directive systems of protection of vertebrate animals for scientific research. Brain cortex was rapidly removed and minced on ice, resuspended in mannitol sucrose HEPES (MSH) buffer (230 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. A protease inhibitor cocktail (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM MSF and 1 μg/ml aprotinin) was added to the homogenates and then centrifuged at 600*g* for 10 min at 4 °C. The supernatant was decanted and centrifuged again at 8000*g* for 10 min; the new mitochondrial pellet was washed several times in MSH without EDTA, in order to avoid calcium chelation by this compound. Mitochondria were stored on ice prior to the experiments. Protein was determined by the Lowry assay (Lowry et al., 1951). The isolated mitochondrial fraction corresponds to synaptic and non synaptic cortex

mitochondria mainly from neurons and glial cells. Previous results from our laboratory have shown the level of cytosolic contamination of our mitochondrial preparations, by measuring the activity of the enzyme lactic dehydrogenase and the microsomal activity antimycin A-insensitive NADH-cytochrome c reductase in mitochondrial fractions, being less than 1.8% and 2.4% of the initial homogenate activity, respectively (Bustamante et al., 2000).

Mitochondrial respiratory function. Oxygen consumption by isolated brain cortex mitochondria was measured with a high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5–1 mg/ml) was placed in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 6 mM malate plus glutamate or 7 mM succinate, 5 mM PO_4H_2K, 4 mM $MgCl_2$ (pH 7.4), and 0.1% free fatty acid bovine serum albumin, at 30 °C. State 3 was estimated by the addition of 0.5 mM ADP. Oxygen uptake was expressed in ng-atom O/min mg protein and the respiratory control ratio (RCR) was calculated from the ratio of the state 3/state 4 respiratory rates with and without ADP, respectively (Estabrook, 1967). The mitochondrial fraction obtained from brain cortex tissue showed a RCR between about 4.0 and 6.0.

Mitochondrial transmembrane potential. Mitochondrial transmembrane potential was determined as follows: isolated mitochondria (25 μg/ml) were incubated at 37 °C for 20 min in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, 1 mM phosphate and 4 mM $MgCl_2$ in the presence of 30 nM $DiOC_6$, a potentiometric probe that can be used for direct measurement of transmembrane potential in cells and isolated mitochondria from different sources. The fluorescence changes were determined by cytometric measurements. Fresh mitochondria were prepared for each experiment and samples were protected from light until acquired by the cytometer. Auto-fluorescence of the mitochondrial preparation was measured as a probe loading control, and 0.5 μM of the depolarizing agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was used as a positive control. Histogram differences in $DiOC_6$ fluorescence were quantified in three independent experiments as the number of events which drop under the median value of the distribution using a common marker (M1) (Bustamante et al., 2011). A higher percentage of $DiOC_6$ fluorescence would reflect mitochondrial membrane depolarization. The RCR was not affected by the concentration of the potentiometric probe used.

Mitochondrial hydrogen peroxide (H_2O_2) production. H_2O_2 generation was determined in intact brain cortex mitochondria by the scopoletin-horseradish peroxidase (HRP) method, following the decrease in fluorescence intensity at 365 and 450 nm as λ exc-λ em at 37 °C (Boveris, 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin and 0.3 μM SOD to ensure that all superoxide anion ($O_2^{\cdot-}$) was converted to H_2O_2; 6 mM succinate plus 6 mM glutamate were used as substrates. Calibration was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min mg protein. H_2O_2 production was highly sensitive to catalase addition (3.500 U/ml).

Evaluation of mitochondrial respiratory complexes I–III, II–III and IV. NADH-cytochrome *c* reductase activity (complex I–III) was measured in brain submitochondrial particles by following spectrophotometrically the reduction of cytochrome *c* at 550 nm (ε = 19.6 $mM^{-1}\,cm^{-1}$) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome *c* and 0.5 mM KCN at 30 °C. Enzyme activity was expressed in nmol cytochrome *c* reduced per minute per mg of protein. Succinate cytochrome *c* reductase activity (complex II + III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate and 1 μM rotenone was added to prevent the possible reverse flux of electrons from complex II to I, yielding ROS formation. Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome c (Yonetani, 1967). The activity was expressed as k'/mg protein.

Western blots. Submitochondrial membranes (150 μg), in the presence of protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin), were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5%), blotted onto a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with rabbit polyclonal antibodies (dilution 1:500) for the two isoforms of nitric oxide synthases: neuronal constitutive form (n-NOS), epitope corresponding to amino acids 2–300, mapping to the amino terminus of NOS I and the inducible constitutive form (i-NOS), epitope mapping to the carboxy terminus of NOS II. Then, the nitrocellulose membrane was incubated with a secondary goat anti-rabbit antibody conjugated with HRP (dilution 1:5000), followed by development of chemiluminescence with the ECL reagent (Santa Cruz Biotechnology) for 2–4 min (Lores-Arnaiz et al., 2007). Voltage-dependent anion channel (VDAC) was used as loading control. Densitometric analysis of neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) neuronaland VDAC bands was evaluated through the Scion Image software (Scion Corporation, Maryland, USA) and expressed as the ratio of NOS isoform/VDAC. All experiments were performed in triplicate.

Nitric oxide production associated with the mitochondria. Nitric oxide production by nNOS and iNOS was measured in brain cortex submitochondrial membranes (0.2–0.5 mg/ml) by using a double-beam dual-wavelength spectrophotometer, following the oxidation of oxyhemoglobin (HbO_2) (25 μM in heme) to methemoglobin at 577–591 nm ($\varepsilon_{577-591}$ = 11.2 $mM^{-1}\,cm^{-1}$), sensitive to L-NNA inhibition (Boveris et al., 2002; Bustamante et al., 2008) at 37 °C. The reaction medium contained 50 mM phosphate buffer (pH 5.8),

50 μM L-arginine, 100 μM NADPH, 10 μM dithiothreitol, 0.5–1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group). In order to assess the production of nitric oxide (NO) by nNOS, 1 mM $CaCl_2$ was added to the reaction medium. Independent activity of iNOS was performed by using a buffer system without calcium, in the presence of 1 mM EDTA and 1 mM EGTA. In order to avoid the presence of O_2^- and H_2O_2, Cu–Zn SOD and catalase at 4 μM and 0.1 μM respectively, were also added to the reaction medium (Lores-Arnaiz et al., 1999). The results were expressed as nmol of NO per minute per milligram protein. NO production associated with the mitochondria, is also strongly dependent on the availability of arginine; this compound is present in brain mitochondria in non-limiting concentrations (Lores-Arnaiz et al., 2004).

Statistical analysis

Results are presented as mean ± SEM. Prior to each analysis, test variables were checked for normality; all data were evaluated by the Kolmogorov–Smirnov test in order to follow a posterior parametric or nonparametric statistical analysis. Behavioral and biochemical results were performed using ANOVA and post hoc Tukey in order to analyze the significance of differences between four groups. SPSS (13.0 version) statistical software was used and a difference was considered to be statistically significant when $p < 0.05$.

RESULTS

Evaluation of melatonin effect on the motor performance during ethanol hangover

The effect of melatonin on a specific task of motor performance at the onset of ethanol hangover was evaluated by the tightrope test (see 'Evaluation of melatonin effect on motor performance during ethanol hangover'). Results are shown in Fig. 1. During ethanol hangover, mice average motor performance was almost 50% lower than controls ($p < 0.01$). Mice receiving melatonin during 7 days before ethanol exposure did not show any significant differences in motor performance as compared with controls. Melatonin treatment alone did not affect motor coordination as compared with controls.

Evaluation of melatonin effect on brain cortex mitochondrial function during ethanol hangover

Mitochondrial respiratory function. Malate–glutamate or succinate-dependent oxygen consumption was measured in state 4 (at rest or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis). The RCR (the most sensitive indicator of mitochondrial oxidative phosphorylation coupling) was calculated as the relationship between state 3 and state 4 respiration rate. Table 1 shows oxygen consumption rates of brain cortex intact mitochondria isolated from control, melatonin pretreated, ethanol hangover and melatonin-pretreated

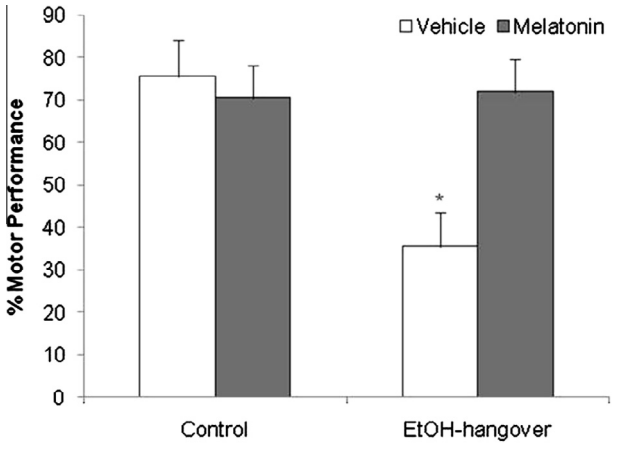

Fig. 1. Tightrope test. Male motor performance during ethanol hangover. Values are expressed as mean ± SEM, $n = 10$ each group. ANOVA, Tukey's test. * $p < 0.01$, vehicle-EtOH-hangover vs. vehicle-control or melatonin-EtOH-hangover. A high percentage represents a better performance in the test. Bar colors indicate pretreatment: white, vehicle; gray, melatonin.

ethanol hangover. Malate–glutamate oxygen consumption of brain cortex mitochondria from melatonin-treated mice showed no differences as compared with control animals for either state 3 or 4. During ethanol hangover, state 3 oxygen uptake was 63% decreased ($p < 0.01$), leading to a reduction in respiratory control of 59%, as compared with control animals ($p < 0.05$). Levels of state 3 respiratory rates were not significantly different from controls in melatonin pre-treated mice before ethanol exposure (Table 1).

Similarly, during ethanol hangover a 24% decrease in succinate-supported state 3 respiratory rate was observed, as compared with controls ($p < 0.01$). RCR was 24% reduced in hangover mice ($p < 0.05$). Melatonin itself induced a 30% decrease in state 4 succinate-dependent oxygen consumption inducing a 39% increase in the respiratory control rate compared with controls ($p < 0.05$). Melatonin pretreatment before hangover did not induce any significant difference in state 3 oxygen consumption compared either with controls or ethanol groups.

Mitochondrial transmembrane potential. The effect of melatonin pre-treatment on transmembrane potential changes that occur in brain cortex mitochondria during ethanol-hangover was evaluated by flow cytometry after loading mitochondria with the potentiometric probe $DiOC_6$. The choice of malate–glutamate as substrates to measure mitochondrial transmembrane potential is based on the fact that substrates for Complex I allow the functioning of respiratory chain with maximal yield of proton pumps. Moreover, according to results from our previous measurements, effects of ethanol hangover were more marked when malate–glutamate were used as respiratory substrates.

Results show a significant decrease in $DiOC_6$ fluorescence median in the ethanol hangover group (8.82) as compared with control group (11.04) (Fig. 2A–D). This fact indicated a clear mitochondrial membrane depolarization by the hangover condition (Fig. 2G).

Table 1. Effect of melatonin on brain cortex mitochondrial oxygen consumption during ethanol hangover

Substrate/Condition	O_2 consumption (ng-atom O/min.mg protein)		
	State 4	State 3	RCR
Mal-Glu			
Control	7.7 ± 0.3	28.2 ± 4.7	3.7 ± 0.4
Melatonin	6.8 ± 1.4	24.6 ± 3.3	3.6 ± 0.2
EtOH-hangover	6.9 ± 1.3	10.4 ± 2.1**	1.5 ± 0.2*
Mel + EtOH hangover	6.1 ± 1.1	23.3 ± 2.6	3.8 ± 0.3
Succinate			
Control	13 ± 1	51.3 ± 2.1	3.9 ± 0.2
Melatonin	9.1 ± 1.3*	48.5 ± 1.6	5.4 ± 0.3*
EtOH-hangover	14.1 ± 1.1	39.1 ± 1.3**	2.8 ± 0.5*
Mel + EtOH hangover	11.3 ± 2.1	43.6 ± 3.2	3.9 ± 0.6

* Malate–glutamate and succinate were used as substrates as described in Materials and Methods. Values represent the mean ± SEM of six to eight individual mitochondrial samples, each obtained from a pool of cerebral cortex from two mice.

** ANOVA Tukey test: *$p < 0.05$; **$p < 0.01$ as compared with control group.

Pretreatment with melatonin before ethanol hangover prevented potential collapse, showing values similar to those from control or melatonin-treated mice (Fig. 2A–F). Melatonin pretreatment before hangover significantly increased $DiOC_6$ fluorescence intensity compared with ethanol hangover condition ($p < 0.05$, Fig. 2G) indicating a preventive mechanism against depolarization as seen in hangover animals. As expected, a significant decrease in $DiOC_6$ fluorescence median was observed after treatment with the depolarizing agent FCCP (Fig. 2F).

Mitochondrial H_2O_2 production. H_2O_2 production rates were determined in the presence of succinate and glutamate in intact mitochondria isolated from the four studied groups. The rationale for the choice of the use of succinate-glutamate to determinate ROS production is based on the fact that these substrates induce the highest production of H_2O_2 (Muller et al., 2008). Results are shown in Fig. 3. Control H_2O_2 production rates were 0.59 ± 0.05 nmol/min mg protein, being significantly decreased by melatonin pretreatment (0.34 ± 0.05, $p < 0.05$). At the onset of ethanol hangover, animals showed a significant increase of 92% in H_2O_2 production as compared with the control group. Melatonin treatment before ethanol hangover shows a 79% decrement of peroxide production as compared with the ethanol hangover group ($p < 0.001$) and a 61% decrement as compared with control animals ($p < 0.05$).

Mitochondrial respiratory complexes I–III, II–III and IV. Respiratory complex activities were measured in brain cortex submitochondrial particles from the different groups. Table 2 showed that all mitochondrial respiratory

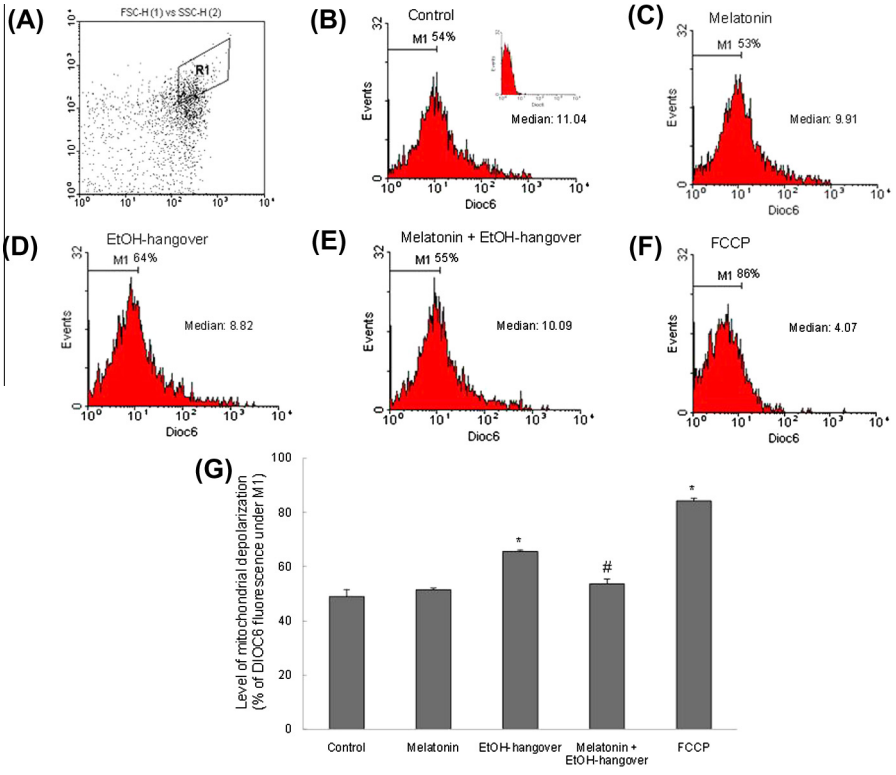

Fig. 2. Evaluation of mitochondrial membrane potential ($\Delta\psi_m$) through the changes in $DiOC_6$ fluorescence intensity. (A) Dotblot of FSC-H vs. SSC-H indicating a gated mitochondrial population (R1). (B–F) Histograms of gated mitochondrial events (R1) vs. relative fluorescence intensity (FL-1) located under marker M1, corresponding to four experimental conditions: (B): controls, (C): melatonin treated, (D): ethanol hangover, (E): melatonin-pretreated ethanol hangover and (F): FCCP-treated mitochondria. (G): Bars scheme of $DiOC_6$ fluorescence quantification representing the mean ± SEM of three different experiments. Fluorescence events were quantified as the number of events which drop under the median value of the distribution using a common marker (M1). ANOVA, Tukey's test (*$p < 0.001$, as compared with control value; #$p < 0.05$, as compared with EtOH-hangover group). Each histogram represents a typical experiment which was performed in triplicate.

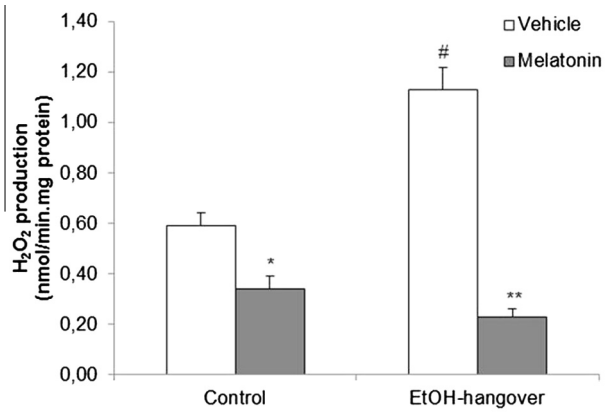

Fig. 3. Effect of melatonin on brain cortex mitochondrial peroxide production rate during ethanol hangover. Mitochondria were supplemented with 7 mM succinate and 6 mM glutamate, as described under Materials and methods. Bars represent the mean ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex of two mice. ANOVA, Tukey's test. $^{*}p < 0.05$, melatonin-control vs. vehicle-control; $^{\#}p < 0.001$, melatonin-EtOH hangover vs. vehicle-EtOH hangover; $^{**}p < 0.001$, vehicle-EtOH hangover vs. vehicle-control. Bar colors indicate pretreatment: white, vehicle; gray, melatonin.

complexes were significantly inhibited during ethanol hangover. Decreases of 19%, 36% and 42% were found in NADH-cytochrome c reductase (Complex I–III), succinate-cytochrome c reductase (Complex II–III) and cytochrome oxidase (Complex IV) activities respectively in brain cortex submitochondrial membranes from the ethanol hangover group. Mice which received melatonin pretreatment before ethanol exposure showed decreases of 56% and 66% in Complex I–III and Complex IV activities, as compared with the control group. At the onset of ethanol hangover Complex II–III was increased by 71% in melatonin-pretreated animals as compared with ethanol hangover group. Interestingly, complex I–III and complex IV activity were lowest in the melatonin-treated hangover condition (Table 2). In addition, a statistical tendency for an increment in complex II–III activity due to melatonin alone was found ($p = 0.058$).

Nitric oxide synthase protein expression. Brain NOS associated with mitochondrial membranes has been previously identified by Western blot analysis mainly as nNOS and also but to a lesser extent as eNOS and iNOS (Lores-Arnaiz et al., 2004). In this study, Western blot analysis for nNOS and iNOS isoforms in association with submitochondrial membranes from cerebral cortex were shown in Fig. 4A. Results showed that nNOS protein expression in brain cortex mitochondria was not different between control and experimental groups (Fig. 4B). On the other hand, iNOS expression was significantly decreased by EtOH-hangover and melatonin pretreatment before hangover prevented this effect. Melatonin alone significantly increased iNOS protein expression, as compared with the control group.

Table 2. Effect of melatonin on enzymatic activity of brain cortex mitochondrial respiratory complexes

Experimental group	Enzymatic activity		
	Complex I–III (nmol/min mg prot)	Complex II–III (nmol/min mg prot)	Complex IV (k'/mg prot)
Control	169 ± 13	44 ± 2	67 ± 3
Melatonin	125 ± 17	51 ± 1	35 ± 8*
EtOH-hangover	137 ± 11*	28 ± 1*	39 ± 8*
Mel + EtOH hangover	74 ± 3*	48 ± 4**	23 ± 3*

Values represent the mean ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex from two mice.
ANOVA Tukey test:
* $p < 0.05$ as compared with control group.
** $p < 0.01$ as compared with EtOH hangover group.

Nitric oxide production associated to the mitochondria. Nitric oxide production by nNOS and iNOS is shown in Fig. 4C. Ethanol hangover induced a 25% decrease in NO production by both NOS isoforms as compared with control mice ($p < 0.05$). Melatonin alone increased NO production by 50% and 25% by nNOS and iNOS respectively ($p < 0.001$; $p < 0.05$). In addition, NO production by nNOS was 25% increased by the pretreatment with melatonin before hangover ($p < 0.05$). Regarding NO production by iNOS, while a decrease in the enzyme expression was evidenced in the melatonin-hangover group, a 50% increase in NO production was also observed, indicating a clear enhancement in the activity of the enzyme despite the low expression.

DISCUSSION

Previous observations by our laboratory have shown a decrease in motor coordination in male mice during ethanol hangover (Bustamante et al., 2012). In the present study, administration of melatonin in drinking water during 7 days before acute ethanol treatment, improved motor performance as tested by a specific neuromuscular coordination task (Fig. 1). In accordance, it has been observed that long-term melatonin treatment significantly prevents locomotor deficits seen in chronic Parkinson's mice (Patki and Lau, 2011). Furthermore, other researchers established that chronic melatonin treatment reverses cognitive deficits in aged and ethanol-intoxicated mice associating this effect to its antioxidant properties (Raghavendra and Kulkarni, 2001). Here, we observed that melatonin turned out to be beneficial in preventing motor impairment when evaluated by one specific behavioral motor task.

We also tested melatonin pretreatment effect on brain cortex mitochondrial function during ethanol hangover (Table 1). As previously reported by our laboratory, state 3 malate–glutamate dependent respiratory rate was significantly decreased at the onset of ethanol hangover (Bustamante et al., 2012). Melatonin pretreatment was able to prevent changes in malate–glutamate-dependent oxygen consumption. Interesting to note is that previous work by Absi et al. (2000) has shown that melatonin produces a weak inhibitory effect on Complex I in rat liver mitochondria, while succinate-linked state 3 respiration was not affected by melatonin. In our study, melatonin by itself significantly decreased succinate-dependent

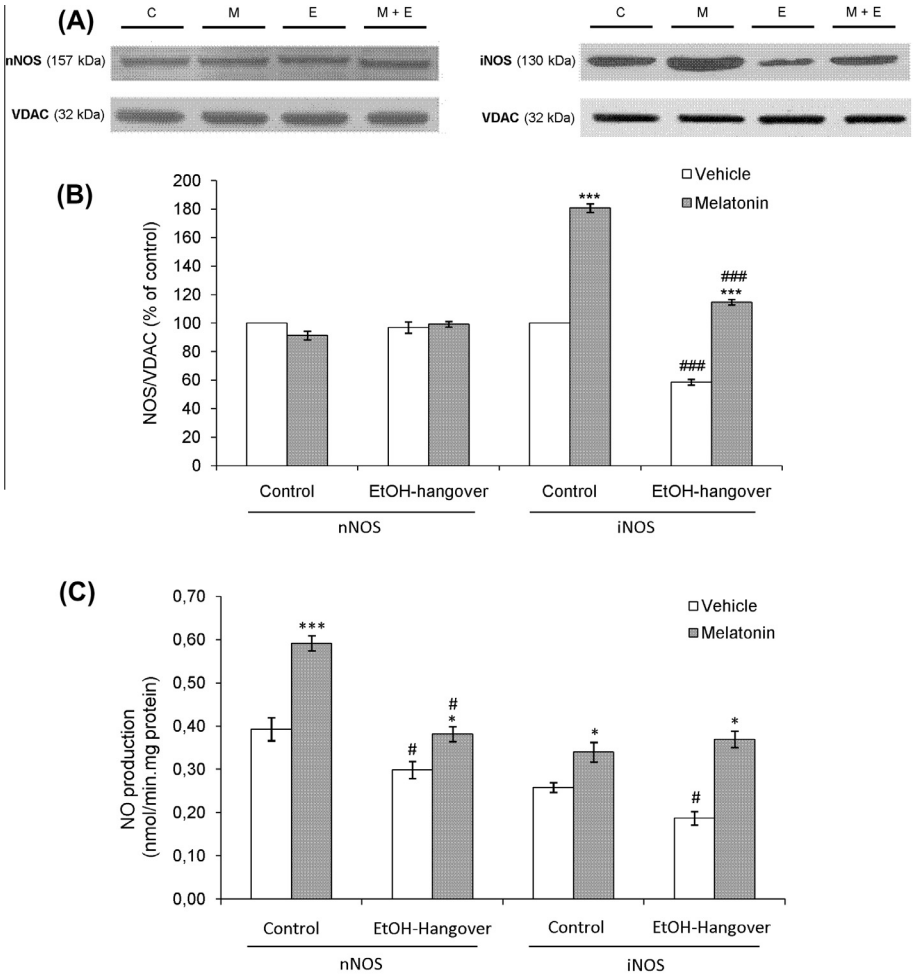

Fig. 4. Effect of melatonin on brain cortex nitric oxide synthase expression and NO production and during ethanol hangover. (A) Western blot analysis of nNOS and iNOS protein expression in brain cortex mitochondria. VDAC was used as loading control. C: control, M: melatonin, E: EtOH-hangover and M + E: Melatonin pretreatment + EtOH-hangover. Results are representative of three independent studies. (B) Bars represent nNOS or iNOS /VDAC ratio obtained after densitometric analysis. Data (mean ± SEM) are expressed as a % of the control group (set at 100%). ANOVA, Tukey's test. $^{***}p < 0.001$: melatonin-control vs. vehicle-control and melatonin-EtOH hangover vs. vehicle-EtOH hangover; $^{\#\#\#}p < 0.001$: vehicle-EtOH hangover vs. vehicle-control and melatonin-EtOH hangover vs. melatonin-control. Bar colors indicate pretreatment: white, vehicle; gray, melatonin. (C) Nitric oxide production by nNOS and iNOS in brain cortex mitochondria. Bars represent the mean ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex of two mice. ANOVA, Tukey's test. NO production by nNOS: $^{*}p < 0.05$: vehicle-EtOH hangover vs. melatonin-EtOH hangover; $^{***}p < 0.001$: melatonin-control vs. vehicle-control; $^{\#}p < 0.05$: melatonin-EtOH hangover vs. melatonin-control, vehicle-EtOH hangover vs. vehicle-control. NO production by iNOS: $^{*}p < 0.05$: vehicle-control vs. melatonin-control and vehicle-EtOH hangover vs. melatonin-EtOH hangover; $^{\#}p < 0.05$: melatonin-EtOH hangover vs. vehicle-EtOH hangover. Bar colors indicate pretreatment: white, vehicle; gray, melatonin.

state 4 respiration by 30%, increasing the respiratory control rate by 39%. Our results are in accordance with previous studies showing that melatonin protects the mitochondria from oxidative damage, reducing oxygen consumption concomitantly with its concentration, and inhibiting oxygen flux in the presence of an excess of ADP (Paradies et al., 2010).

Considering the effect of melatonin on complex activity, it seems that melatonin alone decreases specifically complex I and IV activity without affecting complex III. In melatonin-pretreated ethanol hangover mice, an inhibitory effect on complex I and IV increases the electron transport flux control mainly through complex III. Moreover, even when no significant difference was found for complex III between control and melatonin groups, a statistical tendency for an increment in complex II–III activity was observed ($p = 0.058$). Melatonin and ethanol decrease complex I and IV activities to shift electron transport flux control to complex III so that the inhibition of complex III by ethanol decreases the respiratory rate, while melatonin stabilization of complex III in the presence of ethanol restores the respiratory rate back to control levels.

In addition, according to our results of respiration and complex activity, huge spare respiratory capacities can be observed in both complex I and complex IV since lowering their activities by the combined effects of hangover and melatonin did not significantly decrease the respiratory rate. This is in agreement with Davey et al. (1997) who reported that complex IV activity could be 50–60%

reduced before respiration rates and ATP synthesis were severely compromised.

We also tested the effect of melatonin on mitochondrial transmembrane potential ($\Delta\psi_m$) during the hangover state. Pretreatment with melatonin significantly prevented the reduction in $\Delta\psi_m$ induced by ethanol hangover. Our result is in accordance with previous research in which it was demonstrated that melatonin prevented the oxygen–glucose deprivation induced loss of mitochondrial membrane potential, probably by inhibiting the mitochondrial permeability transition (MPT) pore (Andrabi et al., 2004).

Melatonin has been shown to have an important role in protecting cell membranes from oxidative stress, mainly through its oxygen free radical-scavenging properties (Reiter et al., 1999). In the present work, a significant decrease in H_2O_2 production was observed in melatonin-treated mice and in those who received melatonin as a pretreatment before ethanol exposure (Fig. 3). The removal of oxygen radical species by melatonin would contribute to preserve mitochondrial membrane permeability in the ethanol hangover condition. These results are in accordance with other researchers who demonstrated that melatonin acts as an antioxidant agent reducing oxidative damage (Reiter et al., 2007).

NO is a signal molecule that acts at the CNS as neurotransmitter and neuromodulator, and has been involved in cognitive functions (Kemenes et al., 2002). In our study, ethanol hangover clearly decreased total NO production, mainly by decreasing iNOS expression. Interestingly, in this study, melatonin strongly increased iNOS expression and activity when administered alone or as a pretreatment in alcohol hangover. In these conditions, increased NO production by melatonin could be involved in the inhibition of complexes I and IV observed in our study, due to the inhibition of cytochrome oxidase (Brown, 2001) and of NADH: ubiquinone reductase activity (Riobó et al., 2001) by nitric oxide.

In summary, our results demonstrated that melatonin has beneficial effects which included free radical scavenger activity, reduction in H_2O_2 production, maintenance of mitochondrial transmembrane potential and prevention of motor impairment due to the hangover condition. On the other hand, melatonin simultaneously induced changes in NO metabolism and impairment of the respiratory chain in the presence of succinate together with a decrease of complex IV activity. These facts suggest a dual effect for melatonin actions in brain cortex mitochondria which involves its expected antioxidant actions but also changes in NO metabolism that could account for some of its detrimental effects on the mitochondrial respiratory chain. Side effects of melatonin have been previously reported. For instance, melatonin has been proposed to act as an antioxidant or pro-oxidant according to its dosage (Osseni et al., 2000; Lee et al., 2009). Moreover, pro-inflammatory effects of melatonin have been described in an experimental model of arthritis (Jiménez-Caliani et al., 2005).

Further studies will help to understand melatonin actions on mitochondrial physiology and its involvement in the pathophysiology of ethanol hangover.

CONCLUSIONS

This study shows that weekly exposure to melatonin improved motor coordination in male mice at the onset of ethanol hangover. Beneficial effects of melatonin also included free radical scavenger activity, reduction in H_2O_2 production, and maintenance of mitochondrial transmembrane potential. On the other hand, melatonin simultaneously induced an impairment of the respiratory chain probably due to changes in NO metabolism.

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REFERENCES

Absi E, Ayala A, Machado A, Parrado J (2000) Protective effect of melatonin against the 1-methyl-4-phenylpyridinium-induced inhibition of complex I of the mitochondrial respiratory chain. J Pineal Res 29:40–47.

Andrabi SA, Sayeed I, Siemen D, Wolf G, Horn TF (2004) Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. FASEB J 18:869–871.

Boveris A (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol 105:429–435.

Boveris A, Navarro A (2008) Systemic and mitochondrial adaptive responses to moderate exercise in rodents. Free Radic Biol Med 4:224–229.

Boveris A, Lores Arnaiz S, Bustamante J, Alvarez S, Valdez L, Boveris AD, Navarro A (2002) Pharmacological regulation of mitochondrial nitric oxide synthase. Methods Enzymol 359:328–339.

Brasser SM, Spear NE (2002) Physiological and behavioral effects of acute ethanol hangover in juvenile, adolescent, and adult rats. Behav Neurosci 116:305–320.

Brown GC (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochim Biophys Acta 1504:46–57.

Bruno VA, Scacchi PA, Perez-lloret S, Esquifino AI, Cardinali DP, Cutrera RA (2005) Melatonin treatment counteracts the hyperthermic effect of lipopolysaccharide injection in the Syrian hamster. Neurosci Lett 389:169–172.

Bustamante J, Bersier G, Romero M, Badin RA, Boveris A (2000) Nitric oxide production and mitochondrial dysfunction during rat thymocyte apoptosis. Arch Biochem Biophys 15(376):239–247.

Bustamante J, Czerniczyniec A, Cymeryng C, Lores-Arnaiz S (2008) Age related changes from youth to adulthood in rat brain cortex: nitric oxide synthase and mitochondrial respiratory function. Neurochem Res 33:1216–1223.

Bustamante J, Lores-Arnaiz S, Tallis S, Roselló D, Lago N, Lemberg A, Boveris A, Perazzo JC (2011) Mitochondrial dysfunction as a mediator of hippocampal apoptosis in a model of hepatic encephalopathy. Mol Cell Biochem 354:231–240.

Bustamante J, Karadayian AG, Lores-Arnaiz S, Cutrera RA (2012) Alterations of motor performance and brain cortex mitochondrial function during ethanol hangover. Alcohol 46:473–479.

Cagnoli CM, Atabay C, Kharlamova E, Manev H (1995) Melatonin protects neurons from singlet oxygen-induced apoptosis. J Pineal Res 18:222–226.

Calabrese V, Renis M, Calderone A, Russo A, Reale S, Barcellona ML, Rizza V (1998) Stress proteins and SH-groups in oxidant induced cellular injury after chronic ethanol administration in rat. Free Radic Biol Med 24:1159–1167.

Cardinali DP, Pevet P (1998) Basic aspects of melatonin action. Sleep Med Rev 2:175–190.
Carpentieri A, Díaz de Barboza G, Areco V, Peralta López M, Tolosa de Talamoni N (2012) New perspectives in melatonin uses. Pharmacol Res 65:437–440.
Comporti M, Signorini C, Leoncini S, Gardi C, Ciccoli L, Giardini A, Vecchio D, Arezzini B (2010) Ethanol-induced oxidative stress: basic knowledge. Genes Nutr 5:101–109.
Davey GP, Canevari L, Clark JB (1997) Threshold effects in synaptosomal and nonsynaptic mitochondria from hippocampal CA1 and paramedian neocortex brain regions. J Neurochem 69:2564–2570.
Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. Methods Enzymol 10:41–47.
Fee JR, Sparta DR, Knapp DJ, Breese GR, Picker MJ, Thiele TE (2004) Predictors of high ethanol consumption in RIIbeta knock-out mice: assessment of anxiety and ethanol-induced sedation. Alcohol Clin Exp Res 28:1459–1468.
Gilliam DM, Dudek BC, Riley EP (1990) Responses to ethanol challenge in long- and short-sleep mice prenatally exposed to alcohol. Alcohol 7:1–5.
Giusti P, Franceschini D, Petrone M, Manev H, Floreani M (1996) In vitro and in vivo protection against kainate-induced excitotoxicity by melatonin. J Pineal Res 20:226–231.
Golombek DA, Pévet P, Cardinali DP (1996) Melatonin effects on behavior: possible mediation by the central GABAergic system. Neurosci Biobehav Rev 20:403–412.
Jiménez-Caliani AJ, Jiménez-Jorge S, Molinero P, Guerrero JM, Fernández-Santos JM, Martín-Lacave I, Osuna C (2005) Dual effect of melatonin as proinflammatory and antioxidant in collagen-induced arthritis in rats. J Pineal Res 38:93–99.
Jou MJ, Peng TI, Wu HY, Wie YH (2005) Enhanced generation of mitochondrial reactive oxygen species in cybrids containing 4977-bp mitochondrial DNA deletion. Ann N Y Acad Sci 1042: 221–228.
Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, Wu HY, Chen CC, Hsu LF (2007) Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis. J Pineal Res 43:389–403.
Kemenes I, Kemenes G, Andrew RJ, Benjamin PR, ÓShea M (2002) Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. J Neurosci 22:1414–1425.
Kim CY, Nakai K, Kameo S, Kurokawa N, Liu ZM, Satoh H (2000a) Protective effect of melatonin on methylmercury-Induced mortality in mice. Tohoku J Exp Med 191:241–246.
Kim YH, Lee SH, Mun KC (2000b) Effect of melatonin on antioxidant status in the plasma of cyclosporine-treated rats. Transplant Proc 34:2652–2653.
Kim DJ, Yoon SJ, Lee HP, Choi BM, Go HJ (2003) The effects of alcohol hangover on cognitive functions in healthy subjects. Int J Neurosci 113:581–594.
Lapin IP, Mirzaev SM, Ryzov IV, Oxenkrug GF (1998) Anticonvulsant activity of melatonin against seizures induced by quinolinate, kainate, glutamate, NMDA, and pentylenetetrazole in mice. J Pineal Res 24:215–218.
Lee S, Jadhav V, Ayer RE, Rojas H, Hyong A, Lekic T, Tang J, Zhang JH (2009) Dual effects of melatonin on oxidative stress after surgical brain injury in rats. J Pineal Res 46:43–48.
Lores-Arnaiz S, Coronel MF, Boveris A (1999) Nitric oxide, superoxide and hydrogen peroxide production in brain mitochondria after haloperidol treatment. Nitric oxide 3:235–243.
Lores-Arnaiz S, D'Amico G, Czerniczyniec A, Bustamante J, Boveris A (2004) Brain mitochondrial nitric oxide synthase: in vitro and in vivo inhibition by chlorpromazine. Arch Biochem Biophys 430:170–177.
Lores-Arnaiz S, Bustamante J, Czernizyniec A, Galeano P, González Gervasoni M, Rodil Martínez A, Paglia N, Cores V, Lores-Arnaiz MR (2007) Exposure to enriched environments increases brain nitric oxide synthase and improves cognitive performance in prepubertal but not in young rats. Behav Brain Res 3:117–123.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein Measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
Manda K, Ueno M, Anzai K (2007) AFMK, a melatonin metabolite, attenuates X-ray-induced oxidative damage to DNA, proteins and lipids in mice. J Pineal Res 42:386–393.
Mansouri A, Demeilliers C, Amsellem S (2001) Acute ethanol administration oxidatively damages and depletes mitochondrial DNA in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. J Pharmacol Exp Ther 298:737–743.
Menendez-Pelaez A, Reiter RJ (1993) Distribution of melatonin in mammalian tissues: the relative importance of nuclear versus cytosolic localization. J Pineal Res 15:59–69.
Mollenauer S, Bryson R, Speck C, Chamberlin JR (1992) Effects of exercise on ethanol induced hypothermia and loss of righting response in C57BL/6J mice. Pharmacol Biochem Behav 43:285–290.
Muller FL, Liu Y, Abdul-Ghani MA, Lustgarten MS, Bhattacharya A, Jang YC, Van Remmen H (2008) High rates of superoxide production in skeletal-muscle mitochondria respiring on both complex I- and complex II-linked substrates. Biochem J 15:491–499.
Osseni RA, Rat P, Bogdan A, Warnet JM, Touitou Y (2000) Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG. Life Sci 68:387–399.
Paradies G, Petrosillo G, Paradies V, Reiter RJ, Ruggiero FM (2010) Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. J Pineal Res 48:297–310.
Patki G, Lau YS (2011) Melatonin protects against neurobehavioral and mitochondrial deficits in a chronic mouse model of Parkinson's disease. Pharmacol Biochem Behav 99:704–711.
Peng TI, Yu PR, Chen JY, Wang HL, Wu HY, Wei YH, Jou MJ (2006) Visualizing common deletion of mitochondrial DNA-augmented mitochondrial reactive oxygen species generation and apoptosis upon oxidative stress. Biochim Biophys Acta 1762:241–255.
Raghavendra V, Kulkarni SK (2001) Possible antioxidant mechanism in melatonin reversal of aging and chronic ethanol-induced amnesia in plus-maze and passive avoidance memory tasks. Free Radic Biol Med 30:595–602.
Reiter RJ, Tan D, Kim SJ, Manchester LC, Qi W, Garcia JJ, Cabrera JC, El-Sokkary G, Rouvier-Garay V (1999) Augmentation of indices of oxidative damage in life-long melatonin-deficient rats. Mech Ageing Dev 110:157–173.
Reiter RJ, Tan DX, Manchester LC, Tamura H (2007) Melatonin defeats neurally-derived free radicals and reduces the associated neuromorphological and neurobehavioral damage. J Physiol Pharmacol 58:5–22.
Reiter RJ, Paredes SD, Korkmaz A, Manchester LC, Tan DX (2008) Melatonin in relation to the ''strong'' and ''weak'' versions of the free radical theory of aging. Adv Med Sci 53:119–129.
Riobó NA, Clementi E, Melani M, Boveris A, Cadenas E, Moncada S, Poderoso JJ (2001) Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. Biochem J 359:139–145.
Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR (2000) Significance of melatonin in antioxidative defense system: reactions and products. Biol Signals Recept 9:137–159.
Varlinskaya EI, Spear LP (2004) Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague–Dawley rats. Alcohol Clin Exp Res 28:40–50.
Yonetani T (1967) Cytochrome oxidase: beef heart. Methods Enzymol 10:332–335.

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